Activation of the TRKB receptor mediates the panicolytic-like effect of the NOS inhibitor aminoguanidine

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ARTICLE INFO

ABSTRACT

Nitric oxide (NO) triggers escape reactions in the dorsal periaqueductal gray matter (dPAG), a core structure mediating panic-associated response, and decreases the release of BDNF *in vitro*. BDNF mediates the panicolytic effect induced by antidepressant drugs and produces these effects *per se* when injected into the dPAG. Based on these findings, we hypothesize that nitric oxide synthase (NOS) inhibitors would have panicolytic properties associated with increased BDNF signaling in the dPAG. We observed that the repeated (7 days), but not acute (1 day), systemic administration of the NOS inhibitor aminoguanidine (AMG; 15 mg/kg/day) increased the latency to escape from the open arm of the elevated T-maze (ETM) and inhibited the number of jumps in hypoxia-induced escape reaction in rats, suggesting a panicolytic-like effect. Repeated, but not acute, AMG administration (15 mg/kg) also decreased nitrite levels and increased TRKB phosphorylation at residues Y706/7 in the dPAG. Notwithstanding the lack of AMG effect on total BDNF levels in this structure, the microinjection of the TRK antagonist K252a into the dPAG blocked the anti-escape effect of this drug in the ETM. Taken together our data suggest that the inhibition of NO production by AMG increases the levels of pTRKB, which is required for the panicolytic-like effect observed.

1. Introduction

The periaqueductal gray matter (PAG) is a midbrain structure critically associated with the expression of escape/flight responses. Chemical or electrical stimulation of the dorsal regions of the PAG (dorsolateral and dorsomedial columns) in animals triggers escape reactions that have been associated with panic attacks (Depaulis et al., 1992; Brandão et al., 2008). In humans, stimulation of the dorsal PAG (dPAG) leads to feelings of fear and imminent death (Nashold Jr et al., 1969). As evidenced by Mobbs and colleagues, situations of proximal threat increase midbrain activation in, putatively, the PAG area (Mobbs et al., 2007, 2010).

The neuronal nitric oxide synthase (NOS1 or nNOS) enzyme is highly expressed in the PAG, especially in its dorsolateral columns (Vincent and Kimura, 1992; Onstott et al., 1993). Nitric oxide (NO) can freely diffuse across the cell membrane and act as a retrograde signaling molecule, facilitating glutamate release in several brain regions (Lonart et al., 1992; Lawrence and Jarrott, 1993; Guevara-Guzman et al., 1994). Activation of the NMDA receptor (NMDAr) by glutamate stimulates NO synthesis through calcium influx (Garthwaite et al., 1989), resulting in a positive-feedback response. Injections of NO donors or NMDA-receptor agonists into the dPAG precipitates escape reactions, while local administration of NOS inhibitors or NMDAr antagonists prevents such behaviors (De Oliveira et al., 2001; Miguel and Nunes-de-Souza, 2006; Aguiar and Guimarães, 2009).

Activation of extra-synaptic NMDAr (Vanhoutte and Bading, 2003) or increased production of NO (Canossa et al., 2002) inhibit the synthesis/release of brain-derived neurotrophic factor (BDNF). BDNF,

through activation of tropomyosin-related kinase B receptors (TRKB), regulates neuronal maturation (Cohen-Cory, 2002), synaptic transmission (Figurov et al., 1996), and cell growth and survival (Ceni et al., 2014). It has been proposed that BDNF is also crucial to the effect of antidepressant drugs (Nibuya et al., 1995; Russo-Neustadt et al., 1999; Coppell et al., 2003; Duman and Monteggia, 2006; Molteni et al., 2006; Castrén et al., 2007; Martinowich and Lu, 2008). According to the 'neurotrophic hypothesis', antidepressant drugs increase BDNF levels, especially in the hippocampus and frontal cortex, resulting in synaptic function strengthening and antidepressant/anxiolytic responses (Duman et al., 1997). Antidepressant drugs such as fluoxetine are the first-line treatment for panic disorder patients (Freire et al., 2014).

Of importance to the present study, the microinjection of BDNF into the dPAG inhibited escape responses induced by electrical stimulation of this structure (Casarotto et al., 2010). It also attenuated escape produced by exposure to the open arm of the elevated T maze (ETM) (Casarotto et al., 2015), usually interpreted as indicative of a panicolytic-like effect (for a full description of this later test, see Zangrossi Jr and Graeff, 2014). Moreover, chronic treatment with different antidepressants increased BDNF levels in the dPAG, and this effect was associated with the anti-escape action of these drugs (Casarotto et al., 2015).

Based on these findings, we here hypothesized that the inhibition of NOS would induce panicolytic-like effect by increasing BDNF signaling in the dPAG. To test this hypothesis, we first investigated whether acute or repeated treatment with the NOS inhibitor, aminoguanidine (AMG), would inhibit escape behavior in the ETM or induced by hypoxia. Next, we evaluated whether systemic AMG changes NO, BDNF or TRKB phosphorylation levels in the dPAG. Finally, we checked whether TRKB receptors activation in the dPAG is required for the effect of AMG systemically administered.

2. Methods

2.1. Animals

Male Wistar rats weighing 220–250 g, housed in groups of 6 were kept on a 12 h dark/light cycle (lights on at 07:00 h) at $22 \pm 1\,°C$, with free access to food and water throughout the experiment. The experiments reported in this article were performed in compliance with Brazilian Council for Animal Experimentation (CONCEA; protocol number: 114/2007 and 192/2015), which are based on the US National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Drugs

Aminoguanidine hydrochloride (AMG; Sigma-Aldrich, #396494) was dissolved in sterile saline and injected intraperitoneally (ip) at 1 ml/kg volume. K252a (Sigma-Aldrich, #05288) was dissolved in 0.2% DMSO in sterile saline and microinjected into dPAG at 10 pmol/200 nl volume.

2.3. Apparatus

2.3.1. Elevated T-maze

The elevated T-maze (ETM) consisted of three arms of equal dimensions (50 cm × 12 cm) elevated 50 cm above the floor (Zangrossi Jr and Graeff, 2014). One arm was enclosed by 40 cm high walls, perpendicular to two opposite open arms surrounded by a 1 cm high Plexiglas rim.

Two days before the test the animals were gently handled by the experimenter for 5 min for habituation. Twenty-four hours before the test, the rats were randomly exposed to one of the open arms of the ETM for 30 min, as previously described (Casarotto et al., 2015). For this, a wood barrier was temporarily placed at the intersection between the open and closed arms. This pre-exposition, by shortening the latencies to withdrawal from the open arm during the test, renders the escape task more sensitive to the effects of panicolytic drugs.

The animals were tested in the elevated T-maze 60 min after the last drug or vehicle administration. For the avoidance task, each animal was placed at the distal end of the enclosed arm of the elevated T-maze facing the intersection of the arms. The time taken by the rat to leave this arm with the four paws was recorded (baseline). The same measurement was repeated in two subsequent trials (avoidance 1 and 2) at 30s intervals; during which animals were placed in a Plexiglas cage where they had been previously habituated. For the escape task (performed 30s after avoidance 3), rats were placed at the end of the previously pre-exposed open arm and the latency to leave this arm with the four paws was recorded in 3 consecutive times (escape 1, 2 and 3) with 30s intertrial intervals. The measurements were made by a trained observer blinded to the treatment condition. A cut-off time of 300 s was established for the avoidance and escape latencies. For further details of this test, see Zangrossi Jr and Graeff (2014).

2.3.2. Open field

The open-field test was used to assess whether the drugs used affected the locomotion of tested animals. The test was performed in a Plexiglas circular arena (80 cm diameter), with 40 cm high walls. Independent groups of animals were randomly placed in the center of the circular arena 1 h after the injection for behavioral analysis during 15 min. The total distance traveled was determined by the ANY-MAZE software (Stoelting, USA).

2.3.3. Hypoxia test

The hypoxia chamber consisted of a roof-sealed Plexiglas cylinder (25 cm diameter and 35 cm height) with a removable rubber floor. A flow valve connected to both an air pump and a nitrogen (N_2) cylinder was attached to the chamber. Hypoxia (7% O_2) was produced by N_2 administration at a flow rate of 4.5 l/min during approximately 270 s. The concentrations of both O_2 and CO_2 inside the chamber were monitored throughout the experiment (ML206 Gas Analyzer, AdInstruments, Bella Vista, NSW, Australia) and scanned online with the Power Lab Chart 5 software (AdInstruments, Bella Vista, NSW, Australia).

The animals randomly closed in the chamber and acclimated under atmospheric air (21% O_2) for 5 min. For this purpose, room air was flushed into the chamber at 4.5 l/min flow rate. For the induction of hypoxia, N_2 was flushed into the chamber (4.5 l/min) for approximately 4 min, until O_2 is reduced to 7%, and maintained for 6 min. The animal behavior was recorded throughout the experiment by a video camera. The number of upward jumps during the hypoxic challenge was recorded and analyzed by a trained observer blinded to the treatment condition. This behavior, interpreted as escape attempts from the experimental environment, was used as a panic index (for more details on this test, see Spiacci et al., 2015).

2.4. Stereotaxic surgery and local drug injection

The animals tested in experiment 5 were anesthetized with 2,2,2-tribromoethanol (250 mg/kg, ip) and placed in a stereotaxic frame. A guide cannula made of stainless steel (OD 0.6 mm) was implanted into the brain aimed at the dPAG. The following coordinates from lambda were used (Paxinos and Watson, 2007): AP = 0 mm, ML = +1.9 mm, DV = −3.2 mm. The guide cannula was fixed to the skull with acrylic resin and two stainless steel screws. A stylet with the same length as the guide cannula was fixed in the resin to prevent obstruction.

At the end of the surgery, all animals received an antibiotic preparation (0.3 ml, intramuscular, of benzylpenicillin and streptomycin, Pentabiotico Veterinário Pequeno Porte; Forte Dodge, Brazil), and flunixin meglumine (0.1 ml subcutaneous, Banamine, Schering–Plough, Brazil) for post-surgery analgesia. The animals were left undisturbed for

5 days after the surgery, except for normal handling for cage cleaning.

For drug microinjection, a dental needle (0.3 mm outer diameter) was introduced through the guide cannula until its tip was 1 mm below the cannula end. A volume of 200 nl was injected into the dPAG for 60s using a 10ul microsyringe (Hamilton, USA) attached to a microinfusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheters connecting the syringe to the intracerebral needles was used to monitor the microinjection. The needle was removed 30s after the injection was finished. To confirm the site of injection, after the end of the experiments, all animals were deeply anesthetized with 4% chloral hydrate (1 ml/100 g, ip) and 200 nl of fast green dye was injected through the guide cannula. The brains were removed and sliced for determination of the injection site. Only animals with needle tips inside the dorsolateral PAG were considered for the experimental analysis.

2.5. Sample collection and preparation

2.5.1. BDNF assay

Independent groups of animals were deeply anesthetized with chloral hydrate (4%) and the dorsal dPAG dissected out using punching needles (OD 4 mm). The samples were homogenized in lysis buffer (137 mM NaCl; 20 mM Tris-HCl; 10% glycerol) containing protease inhibitor cocktail (Sigma Aldrich, USA, #P2714) and sodium orthovanadate (0.05%, Sigma Aldrich, USA, S6508). The homogenate was centrifuged (10000 xg) at 4 °C for 15 min and the supernatant collected and stored at -80 °C.

BDNF levels in the dPAG samples were analyzed in duplicate by commercial sandwich ELISA kit (Promega, #G7610, USA) according to the manufacturer's instructions. Briefly, following preincubation with monoclonal anti-BDNF antibody and blockade of non-specific sites, the samples were added to 96-well plates. After the secondary and tertiary antibodies, the developed color intensity was read at 450 nm and compared to a standard recombinant BDNF curve (7.8-500 pg/ml). The BDNF concentration was normalized by total protein content in each sample.

2.5.2. Western-blotting protocol

The levels of TRKB and phosphorylated TRKB (pTRKB) were determined by western blotting (Saarelainen et al., 2003). Briefly, thirty micrograms of total protein content in each sample was separated in acrylamide gel electrophoresis and transferred to a PVDF membrane. Following blockade with 5%BSA in TBST (20 mM Tris-HCl; 150 mM NaCl; 0.1% Tween20) the membranes were exposed to anti-pTRK at Tyr706/707 residues (1:1000, Cell Signaling, #4621, USA), total TRKB (1:1000, Cell Signaling, #4603, USA) or GAPDH (1:2000, Santa Cruz, USA, #sc25778) overnight at 4 °C. After washing with TBST, the membranes were incubated with secondary HRP-conjugated anti-rabbit IgG (1,2000, Santa Cruz, #sc2317, USA). Following washing with TBST and TBS, the membranes were incubated with 4-chloronaphtol (4CN, Perkin Elmer, #NEL300001EA, USA) for color development. The dried membranes were scanned and the intensity of bands relative to pTRKB, TRKB and GAPDH were determined using ImageJ software (NIH, version 1.47, USA). TRKB phosphorylation was normalized by total TRKB levels and expressed as percentage of vehicle-treated group to control unwanted sources of variation.

2.5.3. Quantification of nitrite concentration in PAG

Samples from dPAG were homogenized in ice-cold phosphate buffer (pH 7.4; 0.5 ml) and kept on dark until use (within 5 min). Nitrite concentrations were analyzed in duplicate through an ozone-based reductive chemiluminescence assay as previously described (Feelisch et al., 2002). Briefly, 400ul of dPAG samples were injected in a purge vessel, and approximately 8 ml triiodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid) was added. The triiodide solution reduces nitrites to NO, which is purged by

ozone gas and detected by NO analyzer (Sievers Model 280 NO analyzer, Boulder, CO, USA). The data were analyzed using the software OriginLab (version 6.1).

2.6. Experimental procedure

2.6.1. Experiment 1: effect of AMG acute injection in ETM

The potential panicolytic-like effect of acute AMG treatment was investigate using ETM. At day 1, independent groups of experimentally naive rats were pre-exposed to one of the open arms as described previously. On the 2nd day, the animals received a single administration of AMG (15, 30 or 60 m/kg, ip) or vehicle solution and were tested in the ETM 1 h after the drug injection. This dose range was based on preliminary studies from our group.

2.6.2. Experiment 2: effect of AMG repeated treatment in the ETM, hypoxia-induced escape, and open-field test (OF)

The panicolytic-like effect of repeated AMG was investigate using ETM and hypoxia-induced escape test. Since unspecific motor changes could potentially act as a confounding factor for aforementioned tests, thus motor behavior was investigated in the open-field test. Independent groups of experimentally naive rats were used for each behavioral test. AMG (15, 30 or 60 mg/kg) or vehicle solution was injected ip once a day for 7 days. All the behavioral analysis was conducted 1 h after the last injection of AMG or vehicle solution.

2.6.3. Experiment 3: effect of AMG treatment on BDNF levels in the PAG

In order to investigate if AMG effect could be associated to increased levels of BDNF in dPAG, independent groups of experimentally naive animals received daily ip injections of AMG (15, 30 or 60 mg/kg) or vehicle solution for 1 or 7 days, and dPAG samples were collected as described previously 1 h after the last injection.

2.6.4. Experiment 4: effect of AMG treatment on pTRKB and nitrite levels in the PAG

This experiment was conducted in order to confirm that the drug regimen used was sufficient to modulate NO production and TRKB activity in dPAG. Independent groups of experimentally naive rats received daily ip injections of AMG (15 mg/kg) or vehicle solution for 1 or 7 days. dPAG samples were collected as described above 1 h after the last injection. Since no changes were observed in the levels of GAPDH or total TRKB (data not shown), the intensity relative to pTRKB were normalized by total TRKB and expressed as percent from the control group.

A separate cohort of animals received daily ip injections of AMG (15 mg/kg) or vehicle solution for 7 days. On day 6, half of vehicle-treated animals received AMG, therefore the following groups were: vehicle, AMG-acute, AMG-repeated. 1 h after the last injection, the animals were euthanized for quantification of nitrite levels in the dPAG, as described above.

2.6.5. Experiment 5: effect of intra-dPAG administration of K252a on AMG-induced panicolytic-like effect

To test the possible causal relationship between AMG-induced behavioral effects and the changes in pTRKB levels in the dPAG, experimentally naïve animals with a surgically implanted cannula aiming at the dPAG were treated ip with AMG (15 mg/kg) or saline for 7 days and submitted to the ETM 1 h after the last injection. On days 1, 4 and 7 the animals received intra-dPAG injections of K252a (10 pmol/200 nl) or vehicle solution 10 min before the ip administration of AMG. The final groups were: vehicle-saline; vehicle-AMG; K252a-saline; K252a-AMG.

2.7. Statistical analysis

Parametric tests were preferred, in general, in order to gain statistical power. Exception was made for experiments where there was no

homoscedasticity, no normal distribution, or the measurement scale was not continuous, in such cases non-parametric tests were used. Data obtained in ETM was analyzed by two-way ANOVA, with drug treatments and trials as factors, followed by Newman-Keuls' posthoc, when appropriate. Specifically, for experiment 5, data was analyzed by multivariate ANOVA (factors: trials, systemic treatment, intra-dPAG injection) followed by Newman-Keuls' posthoc. Results obtained in the open field and ELISA were analyzed by one-way ANOVA, followed by Newman-Keuls' posthoc. Nitrite levels in dPAG and hypoxia-induced escape reactions were analyzed by Kruskal-Wallis test, followed by Dunn's posthoc. Western blotting data was analyzed by Mann-Whitney's test. For all experiments $p < 0.05$ was considered statistically significant. SPSS 25.0 and GraphPad Prism 6 software were used for statistical analysis and graphical representation of the data. All images used in the present study (TIF files), as well as the raw data (Excel and Prism files) obtained by our measurements, are openly available in figshare (https://figshare.com/), DOI: https://doi.org/10.6084/m9.figshare.7856825.

3. Results

3.1. Experiment 1: effect of AMG acute injection on elevated T maze (ETM)

The two-way repeated measures ANOVA of avoidance data showed a significant effect of trials [$F_{(2,29)} = 42.45$; $p < 0.05$], but not of treatment [$F_{(3,29)} = 0.36$; NS] or interaction between these factors [$F_{(6,29)} = 0.38$; NS] (Fig. 1). No effect of trials [$F_{(2,29)} = 0.17$; NS], treatment [$F_{(3,29)} = 0.49$; NS] or interaction [$F_{(6,29)} = 0.22$; NS] were found for escape latencies, as seen in Fig. 2a, after acute treatment.

3.2. Experiment 2: effect of AMG repeated treatment on the ETM, open field and hypoxia-induced escape

The analysis of avoidance data showed a significant effect of trials [$F_{(2,24)} = 57.94$; $p < 0.05$], but no effect of treatment [$F_{(3,24)} = 0.72$; NS] or interaction between these factors [$F_{(6,24)} = 1.14$; NS], as shown in Fig. 1b. On the other hand, the analysis of escape latencies data (Fig. 2b) revealed a significant effect of treatment [$F_{(3,24)} = 3.63$; $p < 0.05$], without effects of trials [$F_{(2,24)} = 2.79$; NS] or interaction between these factors [$F_{(6,24)} = 0.89$; NS]. The posthoc test showed that AMG at 15 and 60 mg/kg significantly increased the latency to leave the open arm (Newman-Keuls, $p < 0.05$). For additional experiments, the lower effective dose of 15 mg/kg was chosen.

AMG treatment did not induce locomotor changes in the open field test ($F_{(3,24)} = 0.07$, NS). The mean ± SEM(n) of the total distance traveled, in meters, were vehicle: 22.61 ± 6.73(6); AMG 15 mg:

21.09 ± 4.63(7); AMG 30 mg: 22.38 ± 5.68(6) and AMG 60 mg: 19.63 ± 0.86(6).

The Kruskal-Wallis test showed a significant effect of AMG treatment in the hypoxia test [$H = 7.82$; $p = 0.04$] (Fig. 2c). AMG 15 mg/kg reduced the number of jumps compared to the vehicle-treated group (Dunn's $p < 0.05$).

3.3. Experiment 3: effect of AMG treatment on BDNF levels in the PAG

One-way ANOVA revealed that neither acute [$F_{(3,19)} = 0.96$; NS] nor repeated treatment with AMG [$F_{(3,39)} = 0.34$; NS] significantly changed BDNF levels in the dPAG, (Table 1).

BDNF and TRKB levels are expressed as percentage of the vehicle group. TRKB levels were not assessed in amg 30 and amg 60 groups. No significant difference was found by one-way ANOVA (BDNF analysis) and t-Test (TRKB analysis).

3.4. Experiment 4: effect of AMG treatment on pTRKB and nitrite levels in the PAG

As shown in Fig. 3a, Mann-Whitney's *U* test indicates no significant effect of acute treatment with AMG on pTRKB/TRKB levels [$U = 7.00$; $p = 0.18$] in the dPAG. On the other hand, a significant effect on pTRKB/TRKB levels [$U = 2.00$; $p = 0.03$] was observed after repeated drug treatment (Fig. 3b). Regarding nitrite levels in the dPAG, AMG decreased its levels after repeated but not acute administration (Fig. 3c), as indicated by Kruskal-Wallis [$H = 6.33$; $p = 0.04$], followed by Dunn's multiple comparison test.

3.5. Experiment 5: effect of intra-dPAG administration of K252a on AMG panicolytic-like effect

As shown in Fig. 4b, the MANOVA showed a significant interaction between the systemic injection of AMG, central administration of K252a and trials [$F_{(1,17)} = 5.40$, $p < 0.05$] on the escape behavior in ETM. The post-hoc test showed that in escape 3 the animals in the group vehicle-AMG had longer escape latency when compared to all other groups [$F_{(1,17)} = 8.25$; Newman-Keuls, $p < 0.05$]. Regarding avoidance (Fig. 4a), there was a significant effect of trials [$F_{(1,17)} = 25.9$, $p < 0.05$], but no effect of systemic AMG [$F_{(1,17)} = 0.66$, NS] or K252a intra-dPAG [$F_{(1,17)} = 2.30$, NS]. Also, there was no interaction between the three factors [$F_{(1,17)} = 1.08$, NS]. In this experiment, panicolytic-like effect of AMG appears in escape 3 but not in escape 1 and 2, which differs from what was observed with the experiment presented in Fig. 1b. The reason for this discrepancy is no clear but may be due to a confounding effect of cannulation as an extra variable present in last experiment but not in the first one.
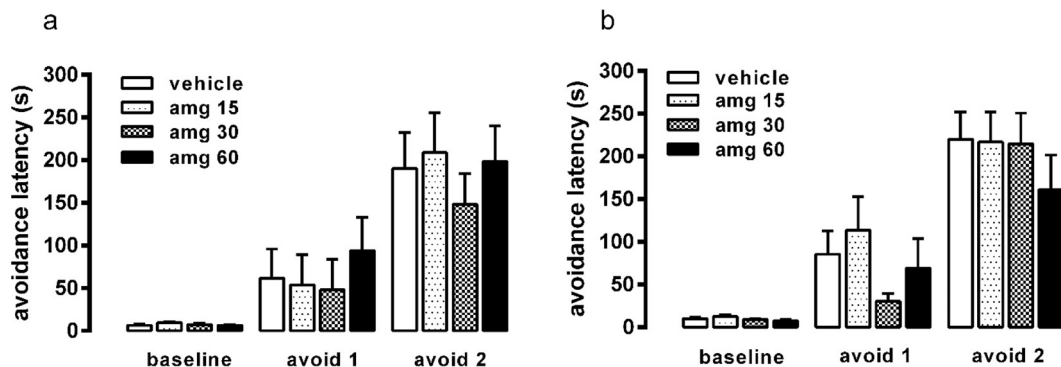

Fig. 1. Effect of acute (left) or repeated treatment (right) with aminoguanidine (amg) in the ETM. Animals received i.p. injections of amg 15, 30 or 60 mg/kg for 1 day (a: n = 8,8,8,9) or 7 days (b: n = 11/group) and the effect upon avoidance latency in ETM was measured. Columns represent mean ± SEM; two-way ANOVA, p > 0.05 (NS).
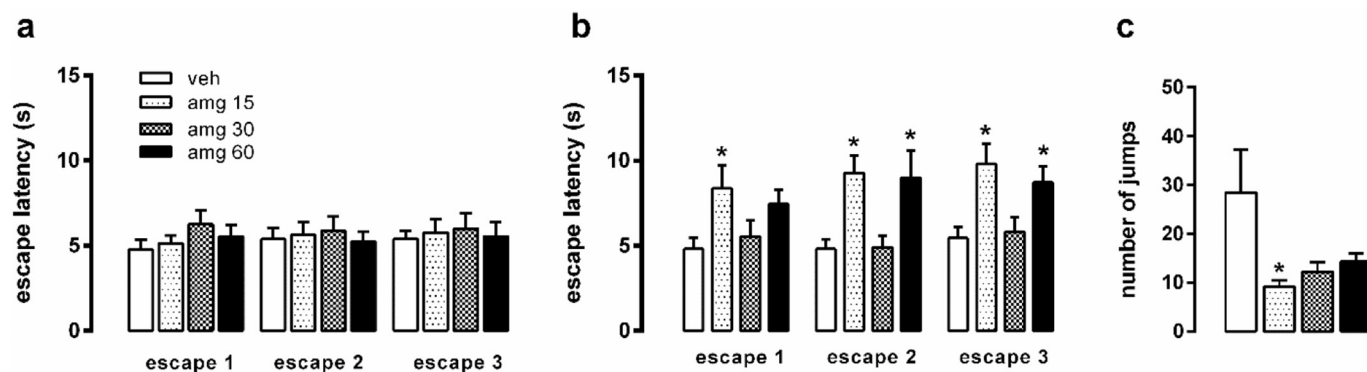
Fig. 2. Repeated treatment with aminoguanidine (amg) induces panicolytic-like effect in the ETM and hypoxia test. Effect of amg after 1 day (a: n = 8,8,8,9) or 7 days treatment (b: n = 11/group) on escape latency in ETM, and in the hypoxia-induced escape reaction (c: n = 7,8,8,8). Columns represent mean ± SEM; two-way ANOVA (a, b) followed by Newman-Keuls' multiple comparison test (when applicable) or Kruskal-Wallis test (c), $*p < 0.05$ *vs* vehicle-treated group at the same trial.

Table 1

Effect of acute and repeated treatment with aminoguanidine (15, 30, 60 mg/kg i.p.) on BDNF and total TRKB levels in dPAG.

	BDNF (mean ± sem)	TRKB (mean ± sem)
Acute treatment		
vehicle	100 ± 14.5	100 ± 12.3
amg 15	103 ± 14.5	134 ± 26.2
amg 30	77 ± 8.7	–
amg 60	88 ± 10.4	–
Repeated treatment		
vehicle	100 ± 13.9	100 ± 10.3
amg 15	109 ± 10.0	92.0 ± 10.0
amg 30	93 ± 13.8	–
amg 60	104 ± 7.6	–

4. Discussion

The present study investigated the effect of the non-selective NO synthase inhibitor AMG on the escape response of rats submitted to the ETM and hypoxia tests, and the involvement of TRKB signaling in the AMG-induced effects.

Aminoguanidine is considered a preferential inhibitor of the inducible isoform of NOS (iNOS or NOS2). However, AMG selectivity for iNOS over nNOS is only 4.7 fold (Wolff et al., 1997). Due to the higher basal expression of nNOS compared to iNOS (Vincent and Kimura, 1992; Onstott et al., 1993), especially in the dPAG, it is very unlikely that in our experimental conditions AMG is acting significantly upon iNOS. We were not able to detect iNOS in dPAG of naïve male rats by immunofluorescence (data not shown). Additionally, raw expression values for nNOS and iNOS, obtained from *in-situ* hybridization experiments scanning the whole brain of mouse through sagittal sections, were extracted from Allen Brain Atlas Data Portal (Lein et al., 2007) and compared. Values for iNOS were close to noise/undetectable in general (raw value range is 0.05–0.56, lowest and highest values were from cortical subplate and olfactory areas, respectively), raw signal from midbrain area was 0.2. Expression of nNOS was substantial, as expected, and lower and highest raw values were obtained for thalamus (1.13) and cortical subplate (5.2), respectively. We also compared nNOS and iNOS expression using Expression Atlas from EMBL-EBI (Papatheodorou et al., 2018) which provides data from RNAseq experiments, and we obtained similar results: nNOS and iNOS average expression in whole brain (in TPM, transcripts per million), respectively, were 27.25 and 0.82. Considering all those pieces of information, our perspective is that aminoguanidine would preferentially inhibit iNOS in a situation where both isoforms are expressed in similar levels (which is the case for the studies that stablished the selectivity of this drug *in vitro*). However, in experimental conditions where iNOS is hardly expressed we would expect that nNOS would be the primary target of aminoguanidine.

AMG inhibited escape response both in the hypoxia test and in ETM. In the hypoxia test, repeated treatment with three doses of AMG decreased escape expression. The effect, however, only reached statistical significance with the lowest dose. The hypoxia test is based on the idea that panic-attacks are a result of a misinterpreted sensation of suffocation, as proposed by Donald Klein (Klein, 1993). In a recent study, Spiacci and colleagues described the role of the dPAG area in mediating escape responses (jumps) induced by hypoxia exposure. In the same way of our results with repeated AMG treatment, these responses were
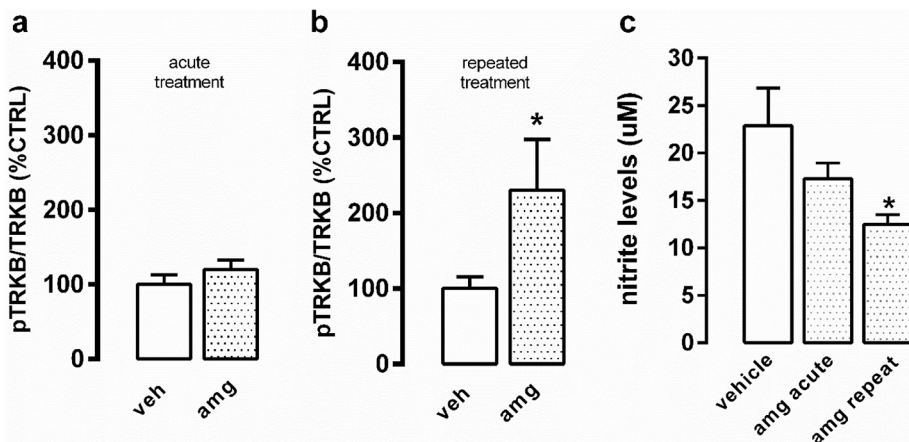

Fig. 3. Repeated but not acute treatment with aminoguanidine increases phospho TRKB and decreases nitrite levels in dPAG. Effect of aminoguanidine (15 mg/kg, ip) acute (a; n = 5,6) or repeated treatment (b; n = 5/group) on TRKB phosphorylation and nitrite levels (c; n = 11,10,9) in the dPAG. TRKB phosphorylation was normalized by total TRKB levels and expressed as percentage of control (vehicle-treated). Columns represent mean ± SEM; Mann-Whitney (a,b) or Kruskal-Wallis followed by Dunn's multiple comparison test (c), $*p < 0.05$ *vs* vehicle-treated group.
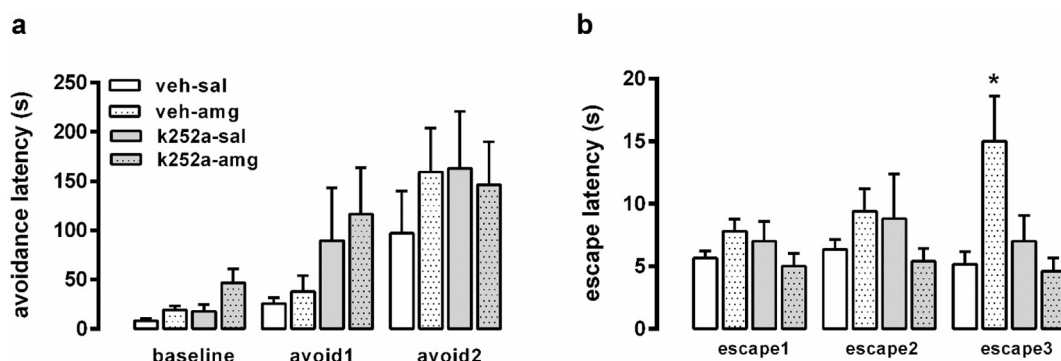
Fig. 4. Aminoguanidine-induced panicolytic effect is abolished by the TRKB antagonist K252a. Effect of aminoguanidine (15 mg/kg i.p.) repeated treatment (7 days) and intermittent intra-dPAG injection of K252 (10 pmol/200 nl, injected at days 1, 4, 7) on avoidance (a) and escape (b) latencies in ETM (n = 6,5,5,5). Avoidance (a) and escape (b) latencies were expressed as mean ± SEM; two-way repeated measures ANOVA, *p < 0.05 *vs* vehicle-treated group at the same trial.

attenuated by high potency benzodiazepines (alprazolam) or repeated treatment with fluoxetine (Spiacci et al., 2015).

In the ETM, repeated but not acute treatment with AMG 15 or 60 mg/kg induced an evident anti-escape effect, which was not observed with 30 mg/kg. The reason for the inefficacy of this intermediate dose is not clear, but similar U- or bell-shaped curves for the effect of NOS inhibitors are not uncommon (Calixto et al., 2008; Lisboa et al., 2013). The frequency of such 'U-shaped' and the hormetic model has been revised by Calabrese and Baldwin (Calabrese and Baldwin, 2003a, 2003b), and appears to be more common than acknowledged. However, little is known about the molecular mechanisms behind these observations (Rozman and Doull, 2003). It has been proposed that under a challenge (for example, drug treatment) several modifications would be triggered in the nervous system to counteract the problem. However, if the given challenge is too mild (eg. drug treatment with low doses), the counteracting response would not be triggered thus allowing the drug effectiveness. On the other hand, if the challenge is too harsh (eg. higher doses) it will exceed the counteracting response, allowing the drug effectiveness once again. In this scenario, an intermediate dose (enough to trigger the counteracting response but not to surpass it) would result in the inefficacy of the treatment.

Another possibility to explain the lack of effect of the intermediary dose of AMG in LTE escape response is that AMG systemically delivered would differentially affect distinct brains areas, depending on the dose used. Despite the nitrite data implicates NOS inhibition in dPAG, it is not possible to rule out AMG-induced NO inhibition in other brain areas, especially at higher doses. This possibility should be investigated in further studies.

Similar to what we have observed with AMG, several clinically used antidepressive/panicolytic drugs, such as fluoxetine, imipramine and escitalopram, decrease escape expression in the ETM. Some of them (e.g imipramine and escitalopram) also impair inhibitory avoidance acquisition, indicating an anxiolytic effect (Pinheiro et al., 2007; Zangrossi Jr and Graeff, 2014). Under the experimental conditions of the present study, however, neither acute nor repeated treatment with AMG was effective regarding this parameter.

Prior studies have investigated the effects of NOS inhibitors in the ETM, but as far as we know, there are no other studies examining the effects of the repeated treatment with these drugs. In agreement with our data, no panicolytic-like effect was observed on escape behavior after a single injection of the NOS inhibitor L-NAME, either i.p. or intra-dlPAG. However, an anxiolytic-like effect on avoidance response was observed by both i.p. or intra-dlPAG injections (Calixto et al., 2001, 2008). The reason for this discrepancy is not clear but it may conceivably reflect the differences in selectivity for the NOS isoforms between AMG and L-NAME. L-NAME systemically delivered induced a significant increase in the blood pressure which indicates an effect upon endothelial NOS isoform. In fact, as described by Boer and colleagues

(Boer et al., 2000), AMG is 5- or 6-times more selective for nNOS or iNOS, respectively, than for eNOS. In the same study, the authors observed that L-NAME exhibit no selectivity between nNOS and eNOS isoforms.

Behavioral changes induced by NOS inhibitors have been described after acute and repeated administration, depending on the animal model. For example, NOS inhibitors induce antidepressant-like effect in the forced swimming test after a single injection in mice (Harkin et al., 1999; Montezuma et al., 2012), while repeated treatment is required to observe similar effects in the chronic mild stress or learned-helplessness models (Yazir et al., 2012; Stanquini et al., 2017). A similar profile has been described for classical antidepressant drugs and there is evidence that different molecular mechanisms might be engaged in the effects induced by acute and repeated treatment (Medrihan et al., 2017). In the ETM, single injections were ineffective and repeated treatment is required for the anxiolytic and panicolytic effect induced by classical antidepressants (Teixeira et al., 2000; Poltronieri et al., 2003; Pinheiro et al., 2007). It has been proposed that the long-term effect of classical antidepressants involves complex neuroplastic alterations in the central nervous system mediated by BDNF-TRKB signaling (Karpova et al., 2011). Following these findings, we hypothesized that regulation of neuronal plasticity might also be required for the long-term anti-depressant- and anxiolytic/panicolytic-like effects of NOS inhibitors.

In the proposed scenario, the panicolytic effect of AMG in the ETM and hypoxia test could involve the activation of TRKB receptors in the dPAG. Recently, our group described the involvement of for BDNF signaling in this region in the panicolytic-like effects caused by anti-depressants (Casarotto et al., 2015). Several antidepressant drugs, with anxiolytic and panicolytic properties, such as fluoxetine, increase the levels of pTRKB following acute or short-term treatment regimen in the hippocampus and prefrontal cortex [for review see (Castrén, 2014)]. In previous study we reported that pTRKB and BDNF levels were found elevated in the dPAG of imipramine-, but not fluoxetine-treated animals, after 3 days of drug administration. The differential effect between these compounds on pTRKB levels was also reflected on behavioral results. Only imipramine exerted panicolytic-like effect in the ETM following this short-term regimen. Both drugs increased pTRKB levels in the dPAG after 21 days of treatment (Casarotto et al., 2015), and were equally effective in the ETM at that time point (Pinheiro et al., 2007). The microinjection of BDNF into the dPAG increased the escape latency in the ETM (Casarotto et al., 2015) without causing any changes in the avoidance task, and this neurotrophin also inhibited the escape response induced by electrical stimulation of dPAG (Casarotto et al., 2010).

Prior studies reported an increase in BDNF levels in the hippocampus *in vivo* following repeated treatment with NOS inhibitors, such as 7NI (Stanquini et al., 2017) and aminoguanidine (Beheshti et al., 2019). However, we did not find any change in BDNF levels in dPAG

after AMG treatment. Despite the difference in the brain area investigated (hippocampus *versus* dPAG), there are other important variances in the experimental protocols that could explain the discrepancies. Beheshti et al. (2019) reported that aminoguanidine was able to modulate hippocampal BDNF levels but the effect of aminoguanidine was assessed only in LPS-treated animals, the effect of aminoguanidine *per se* was not investigated. Additionally, in that study LPS effects were found to be associated to modulation of inflammatory markers, such as IL-6, IL-10 and TNF-α in hippocampus, which strongly indicates LPS induction of iNOS expression by microglia. Additionally, only high doses of aminoguanidine were reported to be effective in reduce NO production (100–150 mg/kg, which is 10 times higher than the dose found effective in our study), which is consisted with LPS-induced iNOS expression. Altogether, the data suggests that aminoguanidine effects in Beheshti's study are most likely mediated by iNOS inhibition, while in our study it seems to be mediated by nNOS inhibition. The kinetics of NO production differs among NOS isoforms: nNOS activation is short-lived and tightly controlled by calcium fluctuations and produces NO in picomolar range concentration, while iNOS-derived NO production can be sustained for a long time since it does not dependent on calcium waves - it is mainly regulated by iNOS expression itself which synthesizes NO at nanomolar concentrations, *i.e.* 1000 times greater than nNOS (reviewed in Davies et al., 1995). Therefore, it would be reasonable to expect different effects emerging from nNOS *versus* iNOS inhibition.

Despite the lack of effect of aminoguanidine upon total BDNF levels, we observed that repeated treatment increased pTRKB in dPAG. The ability of AMG treatment to upregulate pTRK in the dPAG was associated with its effectiveness upon the behavioral response in the ETM. Moreover, the blockade of AMG-induced TRKB signaling in the dPAG by k252a prevented the behavioral effects of AMG on escape latency. Similarly, this parameter was affected by systemic imipramine administration or locally injected BDNF. These effects were also blocked by intra-dPAG administration of k252a (Casarotto et al., 2015).

Taken together our data suggests that AMG exerts its panicolytic-like effect through an increase in pTRKB levels in the dPAG. It is still unclear how NO could modulate the activation of BDNF receptor, but it is plausible to consider that NO regulates local BDNF release in dPAG without changing its total levels. Accordingly, Canossa and co-workers (Canossa et al., 2002) showed that NOS inhibition increased BDNF release in cell culture of hippocampal neurons. Another intriguing possibility is a direct modulation of TRKB function by NO, through nitration and/or nitrosylation. Using an algorithm to analyze putative sites for nitration or nitrosylation [developed by (Xue et al., 2010; Liu et al., 2011)] in TRK receptors, we reported previously that TRKB displays sites with high probability for nitration of tyrosine residues (Biojone et al., 2015). Both mouse and rat mature TRKB receptors are potential targets for nitration at Y90, Y342 and Y816 residues. Interestingly, the Y816 residue is also a well-known target of phosphorylation following TRKB activation either by BDNF or antidepressant drugs, which recruits PLC-gamma pathway (Huang and Reichardt, 2001). Functionally, there is no evidence describing changes in nitrated TRKB responses. However, the phosphorylation of TRKA receptors by NGF prevents its nitration by peroxynitrite, suggesting a competitive functional interaction between these two modifications (Spear et al., 2002). Therefore, inhibiting the production of NO and putatively reducing the probability of TRKB nitration, could facilitate BDNF effect on TRKB receptor in the dPAG, leading to the observed panicolytic-like effect. This is an interesting hypothesis to explain nitric oxide effects on TRKB phosphorylation, which remains to be explored in future investigation.

Overall our study shows that inhibition of physiological levels of nitric oxide modulates TRKB receptor phosphorylation in dPAG. Inhibition of nitric oxide production triggers TRKB activation thus inducing panicolityc-like response. Therefore, our study suggests that imbalanced nitric oxide production and TRKB signaling in dPAG could play a critical role in the pathogenesis of Panic Disorder.

Contributors

The hypothesis was proposed by CB and experimental design was developed by CB and PCC. Data was collected by DER, PCC, CB, ASJ, GGF, LCP, JETS. Data analysis and interpretation was conducted by DER, PCC, CB, HZJ, FSG, SRLJ, CB. All the authors contributed to the manuscript writing and approved the final version submitted.

Conflict of interest and funding

Conflict of interest

None.

Acknowledgements

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